

***Lactobacillus rhamnosus* STRAIN AND USES THEREOF**

BACKGROUND OF THE INVENTION

1. Field of the Invention

5 The present invention relates to a novel lactic acid-forming microorganism which exhibits excellent probiotic properties. The present invention also relates to the uses of the novel microorganism as a probiotic in food, beverage, animal feed and/or dietary supplement compositions, and as a medicament in
10 controlling the colonization of undesirable intestinal microorganisms in the alimentary tract of a mammal.

2. Description of the Related Art

 The oral administration of large numbers of *Lactobacillus rhamnosus*, such as *L. (casei subsp.)*
15 *rhamnosus* GG (ATCC 53103), to a mammal has been found helpful to maintain or even enhance the healthy state of the mammal. It is believed that *L. rhamnosus*, when ingested, would colonize transiently on the intestinal mucosa, which results in inhibition of the growth of
20 pathogenic bacteria and viruses (such as rotavirus), stabilization of gut permeability, and suppression of allergic reactions in food hypersensitivity. The bacterium is particularly effective in alleviating the symptoms of gastroenteric disorders, such as diarrhea,
25 by eliciting nonspecific humoral immune response in hosts.

L. rhamnosus, reported in 1989 as a new species derived

from *L. casei*, shares similar phenotypes with two other members of the *Lactobacillus* genus, i.e., *L. casei* and *L. paracasei*. The three species can be further distinguished in terms of the differences in the genes encoding ribosomal RNAs. Approaches have been conducted based on this finding. For example, Rodtong et al. recognized the species-uniqueness of 16S rDNA and developed a ribotyping process to differentiate *Lactobacillus* strains (Rodtong, S. and Tannock, G. W. (1993) *Applied and Environmental Microbiology* 59: 3480-3484). Taking advantage of the convenience and effectiveness of polymerase chain reaction (PCR), Ward et al. and Alander et al., on the other hand, used different sets of primers to identify *L. rhamnosus* based on the sequence polymorphism of 16S rDNA (Ward, L. J. H., and Timmins, M. J. (1999) *Letters in Applied Microbiology* 29: 90-92; and Alander, M. et al., *Applied and Environmental Microbiology* 65: 351-354).

According to the present invention, the inventor has identified a novel strain of *L. rhamnosus* (hereinafter referred to as strain Tcell-1) which is phylogenetically distinct from the published strains in the species and exhibits excellent probiotic properties.

SUMMARY OF THE INVENTION

It is a primary object of the present invention to provide a novel strain of *L. rhamnosus*. In the

experiments performed in the invention, the inventor has characterized the phylogenetic distinction of the bacterial strain and demonstrated the desired probiotic properties thereof.

5 Another object of the present invention is to provide a composition containing the bacterium strain according to the invention and a suitable excipient for the manufacture of foodstuffs, such as beverages, food, animal feed, and dietary supplements.

10 Still another object of the present invention is to provide a pharmaceutical composition comprising the bacterium strain according to the invention, as well as to provide a method for the treatment or prophylaxis of gastroenteric disorders in a subject by administering
15 such a composition to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will become apparent from the following
20 detailed description of this invention, with reference to the accompanying drawings, in which:

Figs. 1A-C demonstrate the enteroscopic sampling from the upper jejunum and rectum tissues of a volunteer;

Fig. 2 is a fermentation profile of the bacterial
25 strain according to the present invention;

Fig. 3A is a restriction map of the chromosomal DNA from the bacterial strain according to this invention;

Fig. 3B shows the result of Southern analysis of Fig. 3A using *E. coli* MRE600 16S+23S rDNA as the probe;

Fig. 4 shows the result of PCR analysis using the primers designed by Ward & Timmins, in which the DNA extracted from the bacterial strain according to the invention (lane 2) and water (lane 3; as a negative control) was subjected to PCR;

Fig. 5 shows the result of PCR analysis using the primers designed by Alander et al., in which two sets of the species-specific primers, rham-rham2 (lanes 2-3) and rham-casei (lanes 4-5) were used in the PCR; and

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, a strain of *L. rhamnosus* was isolated from the intestinal specimens donated by domestic volunteers. In a preliminary process, the microorganisms from the specimens were screened by a series of selective media, among which MRS agar medium and Rogosa SL agar medium exclusively allow the proliferation of *Lactobacillus*. The bacteria selected according to the above procedure were subjected to a four-step screening strategy for identifying *L. rhamnosus*:

Step 1: fermentation patterning using an API 50CHL kit (Biom'erieux, Lyon, France);

Step 2: ribotyping according to the method described in Rodtong et al. (supra), in which the total DNAs

extracted from the microorganisms were treated with restriction enzymes *EcoRI*, *BclI*, *BglII* or *HindIII* and detected by the rDNA probe of *Escherichia coli* subsequent to Southern blotting, so that the restriction fragment fingerprints of the suspected microorganisms can be obtained and compared with those derived from the *L. rhamnosus* DNA;

Step 3: PCR analysis according to the method described in Ward et al. (supra), in which a universal primer Y₂ (5'-CCCAC TGCTG CCTCC CGTAG GAGT-3') and a species-specific primer rham (5'-TGCAT CTTGA TTAA TTTTG-3') were used in the reaction such that a major product of 290 bp will be produced when the chromosomal DNA of *L. rhamnosus* appears in the reaction mixture; and

Step 4: PCR analysis according to the method described in Alander et al. (supra), in which a pair of species-specific primers, rham (as indicated in Step 3) and rham2 (5'-CCGTC AATTC CTTTG AGTTT-3'), will amplify a specific product of 863 bp in the presence of *L. rhamnosus* DNA.

Accordingly, the bacterial strain according to this invention was identified to belong to the species *L. rhamnosus*. However, sequencing of the 863 bp product obtained in the Step 4 leads to a surprising finding that the bacterial strain according to this invention is phylogenetically distinct from all the published strains in the species *L. rhamnosus*.

The bacterial strain thus identified was designated as "*Lactobacillus rhamnosus* Tcell-1" and was deposited in the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan, R.O.C. under the accession number CCRC 910145 (on April 14, 2000). The bacterium was also deposited at the American Type Culture Center (ATCC) with accession number PTA-2406 on August 22, 2000 under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

Further studies concerning the probiotic properties of the strain Tcell-1 were also conducted. The results reveal that the bacterial strain according to the present invention can survive and grow well even in the stringent environment that an ingested bacterium would encounter in the gastrointestinal tracts, including extremely acidic pH and a high level of bile. The ability of *L. rhamnosus* Tcell-1 to resist certain antibiotics is apparently beneficial to administration of the bacterium to a subject who is required to take antibiotics. The superiority of the bacterial strain is further reinforced by its capability of inhibiting the growth of various enterobacteria.

In view of the advantageous properties mentioned above, the bacterial strain according to the present invention is suitable for acting as a probiotic.

According to the present invention, the strain Tcell-1 can be formulated into a broad variety of edible materials, including beverages, such as fluid milk, fermented milk, yogurts, fruit juices and sports drinks; food, such as ice cream, cheese and snacks; animal feed; dietary supplements; and infant formulas. Apparently, it is appreciable to those skilled in the art that the bacterial strain of this invention can be formulated in any suitable form by conventional methods for human or non-human animal's uptake. More preferably, the bacterial strain of this invention is formulated into the edible material in combination with other probiotic organisms, such as *L. acidophilus*, *L. brevis*, *L. casei*, *L. plantarum*, *L. salivarius*, *L. bifidus*, *L. bulgaricus*, *L. causasicus*, *Streptococcus lactis* and other strains of *L. rhamnosus*, or a combination thereof. In addition, *L. rhamnosus* Tcell-1 is preferably formulated together with lactosucrose, chitin, chitosan, manitol, yogurt powder or a combination thereof.

L. rhamnosus Tcell-1 can also be used alone or with other active ingredients as a medicament in controlling the colonization of undesirable intestinal microorganisms in the alimentary tract of a mammal, to alleviate the conditions caused thereby. The composition can be formulated in solution, emulsion, powder, tablet, capsule or other adequate forms for oral administration.

The following examples are given for the purpose of illustration only and are not intended to limit the scope of the invention.

5 **THE PREFERRED EMBODIMENTS OF THE INVENTION**

Example 1: Isolation of *L. rhamnosus* Tcell-1

Six healthy adults, aged from 25-45 and having no addiction to alcohol or smoking or chronic use of a drug, participated voluntarily in this experiment. None of
10 them are vegetarians nor have abnormal dietary habit. The voluntary donors were subjected to fasting for 12 hours before enteroscopic sampling. Three biopsy specimens, each about 2 mm² in size, were picked up from different sites in the upper jejunum and rectum of each donor
15 (Figures 1A-C). The tissue specimens were then washed with physical saline (0.9% NaCl in distilled water) and stored in an ice-cold storage solution (0.9% NaCl, 0.1% Peptone, 0.1% Tween-80 and 0.02% Cysteine) for further analysis. The specimens were treated in an ultrasonic
20 bath for 5 minutes and agitated vigorously for an additional 2 minutes. The obtained suspensions were undiluted or diluted in five- or ten-fold, and aliquots of the preparations were spread on the following solid media to obtain the profiles of enterobacteria contained
25 therein (see also Johansson et al., *Applied and Environmental Microbiology* 59(1): 15-20).

1. Brain heart infusion agar (purchased from Difco),

which is an enriched medium for aerobically or anaerobically culturing the entire population of enterobacteria at 37°C for 3 days;

5 2. MRS agar (Difco) for anaerobically culturing *Lactobacillus* at 37°C for 5 days;

3. Phenol ethanol agar (Difco) for aerobically or anaerobically culturing the Gram(+) bacteria at 37°C for 3 days;

10 4. Azide blood agar (purchased from Oxoid) for aerobically culturing *Streptococcus* at 37°C for 2 days;

5. Slanetz-Bartley agar (Oxoid) for anaerobically culturing *Enterococcus* at 37°C for 2 days;

6. Violet red bile glucose agar (Oxoid) for culturing *Enterobacteriaceae* at 37°C for 2 days;

15 7. Rogosa SL agar (Difco) for anaerobically culturing *Lactobacillus* at 37°C for 5 days; and

8. Reinforced clostridial agar (Difco) for anaerobically culturing *L. bifidus* at 37°C for 5 days.

The results are shown in Table 1.

Table 1

In the upper jejunum specimens:

Name of the Donor	Medium [†]														
	BHIA			MRS			PEA			ABA			SBA		
	1x [†]	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0
JS	M [§]	30	1	0	0	0	0	0	0	M	26	0	52	6	3
HK	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0
V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Name of the Donor	Medium								
	VRBGA			RA			RCA		
	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	0	0	0	0	0	0	M	0	0
JS	0	0	0	0	0	0	M	0	21
HK	0	0	0	0	0	0	0	0	0
V	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0
RG	0	0	0	0	0	0	0	0	0

5 In the rectum specimens:

Name of the Donor	Medium														
	BHIA			MRS			PEA			ABA			SBA		
	1x	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	M	47	6	M	23	1	0	9	0	M	1	0	28	0	0
JS	M	15	1	M	8	0	0	12	0	M	0	1	0	1	0
HK	M	1	0	0	0	0	0	0	0	12	0	0	0	0	0
V	M	0	0	0	0	0	0	0	0	30	0	0	0	0	0
B	M	28	11	M	67	3	0	10	0	M	14	0	M	4	4
RG	M	0	0	0		0	0	0	0	1	0	0	0	0	0

Name of the Donor	Medium								
	VRBGA			RA			RCA		
	1x	5x	10x	1x	5x	10x	1x	5x	10x
JF	M	M	M	0	0	0	M	M	M
JS	M	15	0	0	0	0	M	M	0
HK	0	0	0	0	0	0	M	0	0
V	0	0	0	0	0	0	M	0	0
B	M	M	M	M	0	0	M	M	M
RG	0	0	0	0	0	0	M	0	0

† The abbreviation BHIA represents brain heart agar; MRS represents MRS agar; PEA represents phenol ethanol agar; ABA represents azide blood agar; SBA represents Slanetz-Bartley agar; VRBGA represents violet red bile glucose agar; RA represents Rogosa SL agar; and RCA represents reinforced clostridial agar.

‡ 1X, 5X and 10X are the dilution folds of the bacterial suspensions.

§ The letter M indicates that the number of bacterial colonies on the medium plate is higher than 100.

As shown in Table 1, the profiles of enterobacteria in the upper jejunum and rectum specimens are quite different.

Example 2: Isolation of *Lactobacillus*

From the MRS and Rogosa SL agar media in Example 1, 200 colonies were picked up randomly and transferred separately to fresh MRS agar media containing 1% CaCO₃. After incubation, the colonies surrounded by clear zones were picked up, and each of them was transferred to a basal MRS agar medium supplemented with 1% rhamnose and 0.05% chlorophenol red. Finally, the yellowish colonies, presumably constituted by *Lactobacillus*, were picked up

and further transferred to fresh MRS broth and incubated anaerobically at 37°C for 2 days for further analysis.

Example 3: Identification of *L. rhamnosus* Tcell-1 as a new strain

5

(a) Fermentation pattern

The *Lactobacillus* broth prepared in Example 2 was precipitated, washed with distilled water and resuspended in a defined amount of distilled water. The bacterial
10 suspensions thus obtained were investigated using an API 50CHL kit according to the protocol provided by the manufacturer. Upon this procedure, a strain of *L. rhamnosus* was identified based on the fermentation pattern specific to the species (Figure 2) and designated
15 as *L. rhamnosus* Tcell-1.

Total DNA of the strain Tcell-1 was prepared from a 3 ml culture growing in the mid-log phase according to a conventional method described by Sambrook et al. (Sambrook, J. et al., *Molecular Cloning, A Laboratory*
20 *Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, 1989), and resuspended in 50 µl of TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The DNA solution thus obtained was used in the following analyses for further investigation of the strain Tcell-1.

25 (b) Ribotyping analysis

10 µl aliquots of the DNA were digested by restriction endonucleases, *EcoRI*, *BclI*, *BglII* and *HindIII*, respectively,

for 3 hours. The digested products were loaded into the wells of a 0.8% agarose gel, and electrophoresis was carried out at 5 V/cm for 2 hours. The gel was then stained with ethidium bromide, and an image of the gel was obtained as shown in Figure 3A. The DNAs on the gel were denatured and transferred to a nylon-based membrane (Hybond-N⁺, Amersham) as described by Sambrook et al. (supra). To prepare the probe for Southern analysis, 1 μ l of *E. coli* MRE600 16S+23S rRNA (purchased from Boehringer Mannheim) was used as the template which was amplified via incorporation of [α -³²P]dCTP by AMV reverse transcriptase (Bethesda Research Laboratories) and random primers. Blots were hybridized at 68°C for 16-24 hours in a hybridization solution containing 5X SSC, 1X Denhardt's solution, 1% SDS and 100 mg/ml of Harpin sperm DNA, washed properly to enhance the signal-to-noise ratio, and subjected to autoradiography. As shown in Fig. 3B, the ribotype of the strain Tcell-1 was in perfect agreement with the typical pattern of *L. rhamnosus* as described by Rodtong et al. (supra).

(c) PCR analysis using the Ward & Timmins' primers

To an 1 ml eppendorf, 1 μ l of Tcell-1 DNA harvested in Example 3(a), 1 μ l of primer Y₂, 1 μ l of primer rham, 0.5 μ l of DynaZyme II (Finnzymes Oy) and each dNTP (dATP, dTTP, dCTP and dGTP) at 100 μ M were added. The reaction mixture was added with distilled water to a final volume of 50 μ l and further overlaid with mineral oil. The

reaction mixture was placed in a GeneAmp[®] PCR System 2400 thermocycler (Perkin Elmer) and thermocycled under the following conditions:

	Initial condition:	94°C for 3 min.
5		45°C for 45 sec.
		72°C for 1 min.
	Thermocycling:	94°C for 45 sec.
		45°C for 45 sec.
		72°C for 1 min.
10	Number of thermocycles:	30
	Chain extension:	94°C for 45 sec.
		45°C for 45 sec.
		72°C for 5 min.

Following thermocycling, the amplified products were separated on a 0.2% agarose gel. The gel was stained with ethidium bromide, and a major amplicon of 290 bp was observed on the gel under a UV light source (Fig.4)

(d) PCR analysis using the Alander's primers

The PCR in Example 3(c) was repeated except that the Ward & Timmins' primers were replaced with the rham and rham2 primers designed by Alander et al. (supra). The electrophoresis analysis on a 0.2% agarose gel revealed that the amplified products contain a major band of 863 bp (Fig. 5), which, as described above, was reported to be a critical indicator to identify *L. rhamnosus*.

The experiments conducted in Examples 3(a)-(d) conclude that the biochemical and genetic traits of the

strain Tcell-1 matched with those considered belonging to species *L. rhamnosus*.

(e) Differentiation of strain Tcell-1 from other *L. rhamnosus* strains

5 Using TOPO TA cloning™ kit (Invitrogen), the 863 bp product obtained in Example 3(d) was cloned into a pCR-TOPO™ vector according to the protocol provided by the manufacturer. The resultant plasmid was then introduced into TOP10 One Shot™ electrocompetent cells
10 (Invitrogen) by electroporation. Following proliferation of the transformants in a selective medium, the plasmid was harvested and the 863 bp insert was sequenced.

15 The sequence was used as a query sequence and searched against a nucleotide sequence database in the GenBank (<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?CMD=search&DB=nucleotide>). The sequence alignment shown in Table 2 suggests that the strain Tcell-1 is phylogenetically distinct from all the six *L. rhamnosus*
20 strains available in the GenBank based on the 16S rDNA sequences. The Tcell-1 DNA sequence shown in Table 2, which is 776 bp in size, was designated as SEQ ID No.1.

Table 2

symbol comparison table : geneti.q.dat; gap penalty : 4

		1	10	20	30	40	50
5	TCELL1	TATACACTGGTACCTCCCTAAGTGGGATACATTGAAACAATCTATCCGCATAATCAAGA					
		*** **			* *	* *	* * *
	AF21761	TTGTACACACCGCCC.GTCACACCATGAGAGTTTGTAACA...CCCGAAGCCGGTG					
		*****	*****	*****	*****	*****	*****
	E08782	CTTGTACACACCGCCC.GTCACACCATGAGAGTTTGTAACA...CCCGAAGCCGGTG					
10		* * * *	* * *	* *	**	***	* **** *
	AF18273	CCTTCTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACA...CACGAAACTTTGT					
				* * *** **	**		
	A61362	CTAAGGAAACAGACTGAAAGTCTGACG.....					

15	U32966	CTAAGGAAACAGACTGAAAGTCTGACG.....					

	AF12120	AAGGAAACAGACTGAAAGTCTGACG.....					
			1	10	20		
	consens	A C	CC A	GA ACAGAC	GAAA TCT AC	C C	A
20							
		1	10	20	30	40	50
			70	80	90	100	110
	TCELL1	CCGCATGTCTTGCTAAGATGCGTAACTATCGCTTTGGATGACCCCGGTATAGCTAGTTG					
25		** *	*	* * * *		** *	*
	AF21761	GCGTAA.....CCCTTTTAGGGAGCGA.....GCCGTCTAAGGTGGGACAA					
		*****	** ***	**		*	* * * *
	E08782	GCGTAA.....CCCTTTTAGGGAGCGAG.....CCGTCTAAGGTGGGACAAA					
			**	*		*	
30	AF18273	TTAGTTTTGAGGGGATTACCCCTCAAGCACCC.....TAGCGGGTGGGACTTTGTTC					
	A61362					
	U32966					
35	AF12120					
	consens						

Table 2 (continued)

		70	80	90	100	110	120
		130	140	150	160	170	
5	TCELL1	TAAGTAACGCTCACCAAGCAATGATGCTAGCCAACTAAGTTGATCGCCACATTGGACTAA					
		* * * * *	* * * * *	* * *		* * *	* * *
	AF21761	ATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCC					
		* * *	*****				
	E08782	TGATTAGGGTGAAGTCGT.AACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCC					
10		* * * * *		* * *	* * *	* * *	* * *
	AF18273	TTTGAAAACCTGGATATCATTGTTGTAAATGTTTTAAATTGCCGAGAACACAGGCTATTTG					
	A61362					
15	U32966					
	AF12120					
	consens	A		A		C	T
20							
		130	140	150	160	170	180
		190	200	210	220	230	
	TCELL1	ACACGGCC.CAAACTCTACGGAGGCAGCAGTAGGAATCTTCCACAATGGACGCAAGTCTG					
		*	****	* * *		* **	* *
25	AF21761	TTTCTAAG.GAAACAG.ACTGAAAGTCTGA.....CGGAAACCTGCACACACGA					
		*****	*****	*****		*****	
	E08782	TTTCTAAG.GAAACAG.ACTGAAAGTCTGA.....CGGAAACCTGCACACACGA					
		* *	* * *	* * *		* * * * *	* * * *
30	AF18273	TATGAGTTTCTAATAATAGAAATTGCGCAT.....CGCATAACCGCTGACGCAA					
						* * * * *	* * * *
	A61362GAAACCTGCACACACGA					

	U32966GAAACCTGCACACACGA					
35						*****	
	AF12120GAAACCTGCACACACGA					
						30	40
	consens	AA		A	A	C GAAACCTGCACACACGA	

Table 2 (continued)

		190	200	210	220	230	240
		250	260	270	280	290	
5	TCELL1	ATGGAGCAACGCCGCTGACTGAAGAAGGCTTTCGGGGCGTAAAACTCTGTTGTTGGAGA					
		*		** *	*	*	**
	AF21761	AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCTAGCGGGTG.....CGACT					
		*****	*****	*****	*****	*****	*****
	E08782	AACTTTGTTTAGTTTTGAGGGGATCACCTCAAGCACCTAACGGGTG.....CGACT					
10		* * *	* * *	* * *	**		***
	AF18273	GTCAGTACAGGTTAAGTTACAAAGGGCGCACGGTGGATGCCTTGCCACTAGGAGCCGATG					
		* * *	* * *	* * *	**		
	A61362	AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCTAGCGGGTG.....					
		*****	*****	*****	*****	*****	*****
15	U32966	AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCTAGCGGGTG.....					
		*****	*****	*****	*****	*****	*****
	AF12120	AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCTAGCGGGTG.....					
		50	60	70	80	90	
	consens	AACTTTGTTTAGTTTTGAGGGGATTACCCTCAAGCACCTAGCGGGTG					GA
20							
		250	260	270	280	290	300
		310	320	330	340	350	
25	TCELL1	AGAATGGTCGGCAGAGTAAGTGTTCGGCGTGACGGTATCCAACCAGAAAGCCACGGCT					
			*****		**	*	* * *
	AF21761	TTGT.....TCTTTGAAAAGTGGATATCATTGTTGTAATGTTTTAAATTGCCGAGAACA					
		****	*****	*****	***	*****	*****
	E08782	TTGT.....TCTTTGAAAAGTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACA					
		*	** *	* *	** *	**	* * *
30	AF18273	AAGGACGGAACATAACCGATATGCTTCGGGGAGCTATAAGTAAGCTTTGATCCGGAGAT					
				** *		*	*
	A61362CGACTTTGTTCTTTGAAAAGTGGATATCA					
		*****	*****	*****	*****	*****	*****
	U32966CGACTTTGTTCTTTGAAAAGTGGATATCA					
		*****	*****	*****	*****	*****	*****
35	AF12120CGACTTTGTTCTTTGAAAAGTGGATATCA					
				100	110		
	consens		T	GACT T T CTTT AAAA TCGA A CA			

Table 2 (continued)

		310	320	330	340	350	360
		370	380	390	400	410	
5	TCELL1	AACTCAGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG					
		*	*		*	*	
	AF21761	CAGCGTATTTGTATGAGTTTCTAATAA.....TA					
		*****	**	**	**		*
	E08782	CAGCG.TATTTGTATGAGTTTCTGAAA.....AA					
10		**	*	*	*	*	
	AF18273	TTCCGAATGGGGGAACCCAGTACACATCAGTGTATT.....GC					
		**					
	A61362	TTGTTGTAA.....AT					
		*****					**
15	U32966	TTGTTGTAA.....AT					
		*****					**
	AF12120	TTGTTGTAA.....AT					
							130
	consens	TTGT	TA				A
20							
		370	380	390	400	410	420
		430	440	450	460	470	
25	TCELL1	CGTAAAGCGAGCGCAGGCGGTTTTTTAACTCTGATGTGAAAGCCCTCGGCTTAACCGAGG					
		*	*	****		** * **	* *
	AF21761	GAAATTTCGCATCGCA.....TAACCGCTGACGCAAGTC....					
		*****				*****	
	E08782	GAAATTTCGCATCGCA.....TAACCGCTGACGCAAGTC....					
		*	**		** *	*	
30	AF18273	CTGCAAGTGAATACA.....TAGCTTGTGGCGGCAGACGCG					
		*	*	*			
	A61362	GTTTTAAATTGCCGA.....					

	U32966	GTTTTAAATTGCCGA.....					
35		*****					
	AF12120	GTTTTAAATTGCCGA.....					
	consens	GTT	TA	AGC	CA	A	T

Table 2 (continued)

		430	440	450	460	470	480
		490	500	510	520	530	
5	TCELL1	AAGTGCATCGGAAACTGGGAAACTTGAGTACAGAAGAGGACAGTGGAAGTCCATGTGTAG					
		* ** *	*	*	** *	*	*
	AF21761	AGTACCAGGTAAGTTACAAAGGGCGCACGGTGGATGCCTTGGCACTAGGAGC.....C					
		*****	*	*****			*
	E08782	AGTACAGGTTAAGTTACAAAGGGCGCACGGTGGATGCCTTGGCACTAGGAGC.....C					
10		* * * * *	**	**** *	*	* * *	*
	AF18273	GGGAACTGAAACATCTCAGTACCCGCAGGAAGAGAAAGAAAAGTTCGATTCCCATAGTAGC					
		**** *	*	** *	* ** *		
	A61362	..GAACACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATC.....					

15	U32966	..GAACACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATC.....					

	AF12120	..GAACACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATC.....					
		150	160	170	180	190	
	consens	GAACA AG	ATT G A G	T ACTAA	AATA	AA C C A	C
20							
		490	500	510	520	530	540
		550	560	570	580	590	
25	TCELL1	CGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTA					
		*	* *	*** **	* * * *	*	*
	AF21761	GATGAAGGACGGAAGTAAATACCGATATGCTTCGGGGAGCTATA.....A					
		*****					*
	E08782	GATGAAGGACGGAAGTAAATACCGATATGCTTCGGGGAGCTATA.....A					
		* ** * *	*	** * *	*	***	
30	AF18273	GGCGAGCGAAGTGGGAAGAGCCCAAACCGAGAAGCTTGCTTCTCGGGGTGTAGGACTGG					
		* * * * *	**	*	*		
	A61362GCATAACCGCTGACGCAAGTCAGTACAGG					

	U32966GCATAACCGCTGACGCAAGTCAGTACAGG					

35	AF12120GCATAACCGCTGACGCAAGTCAGTACA					
			200	210	218		
	consens		GCATAA	CGCA	ACGCA	G GT CA	

Table 2 (continued)

		550	560	570	580	590	600
		610	620	630	640	650	
5	TCELL1	ACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCAT					
		* ** *	*		*	* **** *	**
	AF21761	GTAAGCTTTGATCCGGAGATT.....TCCGAATGGGGGAACCCAGTA....CAC					
		*****			*****		***
	E08782	GTAAGCTTTGATCCGGAGATT.....TCCGAATGGGGGAACCCAGTA....CAC					
10		*	** ** *		* * * *	* * *	*
	AF18273	ACATTGGAGTTACCAAAGTTCG.....ACGTAGTCGAAGTCAGCTGGAAAGCTGC					
	A61362						
15	U32966						
	AF12120						
	consens		C			C G	
20							
		610	620	630	640	650	660
		670	680	690	700	710	
25	TCELL1	GCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCACTAACGC					
		*	* *** *	** *	* *		* ****
	AF21761	ATCAGTG.....TATTGCCTGCAAGTGAATACATAGCTTGT.....TGGCGGCAGACGC					
		*****	* *	** *	* *		* * *
	E08782	ATCAGTGTGTTGCTTGTTCAGTGAATACATAGCTGGCCGGCG.....GCCAGACGCGGGG					
		** *	* ** * *	* * *			* *
30	AF18273	GCCATAGAAGGTGAAAGCCCTGTAAACGAAACGGCGGACTC....TCCGTCCAGGATCCT					
	A61362						
	U32966						
35	AF12120						
	consens	C		C			

Table 2 (continued)

		670	680	690	700	710	720
		730	740	750	760	770	776
5	TCELL1	ATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG					
		* *		** * **			
	AF21761	GGGGAAC.....GAAACATCTAAG					
		*		* * **			
	E08782	AACTGAAA.....CATCTAAGTACCCGGA					
10		* *		* * * *			
	AF18273	GAGTACGGCGGAACACGTGAAATTCCGTGGAATCCGGGAGGACCATCT					
	A61362						
15	U32966						
	AF12120						
	consens			A	A		
20	~	730	740	750	760	770	778

Example 4: Characterization of *L. rhamnosus* Tcell-1

(a) Tolerance of acid

MRS liquid media were prepared at pH 2, 3, 4, 5 and 6, respectively, and supplemented with 0.3% bile salt. To 1.5 ml of each medium, 10^6 Tcell-1 cells were inoculated and incubated anaerobically at 37 °C. Samples were collected at 0 and 4 hours after the inoculation, and the populations of the microorganisms in each culture were assessed with reference to the optical density at 620 nm. The results are shown in Table 3.

Table 3

Incubation time	OD ₆₂₀				
	pH2	pH3	pH4	pH5	pH6
0 hour	0.01	0.018	0.010	0.014	0.012
4 hour	0.01	0.018	0.016	0.025	0.030

As shown in Table 3, the growth rate of *L. rhamnosus* Tcell-1 remarkably reduced as the cultures were acidified to a pH at which the ingested substances would encounter in an animal stomach, i.e., a pH of below 3. Surprisingly, the cells incubated in such an acidic environment for 4 hours can still restore their normal growth if transferred to a fresh MRS medium at pH 6.0 (data not shown). The data indicate that *L. rhamnosus* Tcell-1 can tolerate the attack of gastric acid.

(b) Tolerance of bile salt

Example 4(b) was repeated except that the MRS liquid

media contained bile salts at concentrations of 0.1, 0.2, 0.3 and 0.4%, respectively, while the pH of the media was constantly set at 2.5. The results are shown in Table 4.

Table 4

Incubation time	OD ₆₂₀			
	0.1%	0.2%	0.3%	0.4%
0 hour	0.011	0.010	0.013	0.018
4 hour	0.028	0.025	0.023	0.032

From Table 4, it is demonstrated that the growth of *L. rhamnosus* Tcell-1 was sustained at a high level of bile.

(c) Resistance to antibiotics

2-3 ml aliquots of a bacterial suspension from an overnight culture of *L. rhamnosus* Tcell-1 were spread on MRS agar media added with 10 μ g/ml of kanamycin, vancomycin, chloramphenicol or ampicillin. After incubation, *L. rhamnosus* Tcell-1, while its growth was attenuated in the media containing chloramphenicol or ampicillin, was found to be tolerant of kanamycin and vancomycin.

(d) Inhibition of the colonization of other bacteria

2-3 ml aliquots of a bacterial suspension from an overnight culture of *L. rhamnosus* Tcell-1 were spread on MRS agar media. Each of the plates was incubated at 30°C for 22 hours, on which 7 ml of soft agar mixed with 100 μ l suspension from one of the nine enterobacterial strains listed in Table 5 (purchased from the FIRDI) was

poured.

Table 5

Bacterium	Medium
<i>Enterobacter aerogenes</i>	DIFCO 0001
<i>Clostridium perfringens</i>	brain heart infusion (anaerobically cultured)
<i>Klebsiella pneumoniae</i>	DIFCO 0001
<i>Yersinia enterocolitica</i>	brain heart infusion
<i>Listeria monocytogenes</i>	brain heart infusion
<i>Streptococcus mutans</i>	brain heart infusion
<i>Citrobacter freundii</i>	DIFCO 0001
<i>Shigella dysenteriae</i>	DIFCO 0001
<i>Yersinia ruckeri</i>	DIFCO 0001

5 The obtained cultures were incubated for an additional
48 hours at 37°C and observed with bare eyes. Based on
the presence of inhibition rings around the colonies of
L. rhamnosus Tcell-1, the inventor found that the strain
Tcell-1 can significantly suppress the growth of *E.*
aerogenes, *C. perfringens*, *L. monocytogenes*, *S. mutans*
10 and *C. freundii*. The results strongly suggest that *L.*
rhamnosus Tcell-1 exhibits promising probiotic
properties for controlling or inhibiting the colonization
of the undesired bacteria in the bowel.

15 Example 5: PROBIOTIC FORMULATIONS CONTAINING *L. rhamnosus*
Tcell-1

L. rhamnosus Tcell-1 can be utilized in various forms
of foodstuffs, two examples of which are described as
follows:

20 Formula 1:

Ten strains of lactic acid-forming bacteria:

L. acidophilus, *L. brevis*, *L. casei*, *L. plantarum*, *L. salivarius*, *L. bifidus*, *L. bulgaricus*, *L. causasicus*, *Streptococcus lactis* and *L. rhamnosus* Tcell-1;

5 Excipients:

lactosucrose oligo, manitol, chitin & chitosan,
yogurt powder;

Natural condensates:

alfalfa, barley and wheat grass juice powder, pure
10 soya lecithin, carrot juice powder, phosphatidyl
choline, Hawaiian *Spirulina pacifica*, apple pectin
powder, phosphatidyl inositol CGF chlorella, non-dairy
probiotic culture: *rhamnosus*, *acidophilus* in a base of
FOS, peace river bee pollen powder, stevia, freeze-
15 dried mango, black currant, dandelion root extract 4:1,
beetroot extract, Siberian ginseng extract 0.4%,
pacific kelp 4:1 extract, artichoke 4:1 extract 2%, soya
extract, bilberry extract 5:1, pineapple extract,
cranberry juice extract 18:1, rosehip extract 4:1,
20 lycopene, Milk Thistle Phytosome™, Ginkgo Biloba
Phytosome™ and Grape Seed Phytosome™;

Other ingredients:

Vitamin C, calcium, magnesium, zinc.

25 Formula 2:

Nine strains of lactic acid-forming bacteria:

L. acidophilus, *L. brevis*, *L. casei*, *L. plantarum*,

L. salivarius, *L. bifidus*, *L. bulgaricus*, *L. causasicus*
and *L. rhamnosus* Tcell-1;

Other ingredients:

5 calcium lactate, lactosucrose oligo, lactose,
dextrose, powered milk, vegetable oil and small amounts
of an emulsifier and natural seasonings

The formulation is coated on peanut and raisin
granules to make up a probiotic healthy dessert.

10 With this invention thus explained, it is apparent
that numerous modifications and variations can be made
without departing from the scope and spirit of this
invention. It is therefore intended that this invention
be limited only as indicated by the appended claims.

15